FORM PTO-1390 (REV 10-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
33339/242251	
U S APPLICATION NO (If known, see 37 C F R 1 5)	
10/019872	

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/01946	July 6, 2000	July 6, 1999

TITLE OF INVENTION

Method For The Production Of An Immunostimulant Milk Product And Uses Thereof

APPLICANT(S) FOR DO/EO/US

Jean-Pierre Blareau; Marie-Benediete Romond; Charles Romond; Francis Lecroix; Charles Gontier

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☒ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO (If known, see 37 C F R 1.50) 10/019872	INTERNATIONAL APPLICATION NO PCT/FR00/01946	ATTORNEY'S DOCKET NUMBER 33339/242251

17. ☒ The following fees are submitted:		CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,040.00**		
International preliminary examination fee (37 CRF 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$ 890.00**	**$ 890.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37CFR 1.445(a)(2)) paid to USPTO	**$ 740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO But all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$ 710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$ 890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		**$ -0-**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	10 -20 =	0	**X $18.00**	**$ 0.00**	
Independent Claims	1 - 3 =	0	**X $84.00**	**$ 0.00**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			**+ $280.00**	**$ -0-**	
TOTAL OF ABOVE CALCULATIONS =				**$ 890.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by one-half.				**$ -0-**	
SUBTOTAL =				**$ 890.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				**$ -0-**	
TOTAL NATIONAL FEE =				**$ 890.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$ -0-**	
TOTAL FEES ENCLOSED =				**$ 890.00**	
				Amount to be Refunded	**$**
				Charged	**$**

a. ☒ A check in the amount of $ 890.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 16-0605 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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Date of Deposit: December 26, 2001

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Lorna Morehead

#5

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re:	Jean-Pierre Blareau, et al.	Attn: DO/US
International Appl. No.:	PCT/FR00/01946	
International Filing Date:	July 6, 2000	
U.S. Application No.:	10/019,872	
For:	Method For The Production Of An Immunostimulant Milk Product And Uses Thereof	

Box PCT
Commissioner for Patents
Washington, DC 20231

March 7, 2002

PRELIMINARY AMENDMENT

Sir:

Please add the following new Claim 10:

In The Claims:

10. (New) The *Bifidobacterium breve* strain I-2219 deposited at the CNCM on May 31, 1999.

REMARKS

The subject matter of this newly added claim is described in the specification at page 4, lines 7-25.

Respectfully submitted,

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CERTIFICATE OF EXPRESS MAILING

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Lorna Morehead

CLT01/4523422v1

Attorney's Docket No. 33339/242251

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Jean-Pierre Blareau, et al.
Appl. No.: To be assigned Group Art Unit: To be assigned
Filed: Concurrently herewith
For: METHOD FOR THE PRODUCTION OF AN IMMUNOSTIMULANT MILK PRODUCT AND USES THEREOF

December 26, 2001

Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Dear Sir:

Please amend the above-identified application as follows:

Please add the following Abstract of the Disclosure:

METHOD FOR THE PRODUCTION OF AN IMMUNOSTIMULANT MILK PRODUCT AND USES THEREOF

A method for the production of an immunostimulant milk product, characterized in that bioconversion is carried out on or a milk substrate with the aid of a *Bifidobacterium* culture by keeping said substrate in contact with said culture in conditions which are unfavorable with respect to the fermentation of *Bifidobacterium*. The invention also relates to milk foods and products obtained by said method.

In The Specification:

Page 1, after the title and before the first paragraph (numbered line 4) please insert:

FIELD OF THE INVENTION

Page 1, between the first and second paragraphs (numbered lines 8 and 9) insert:

BACKGROUND OF THE INVENTION

Page 2, between numbered lines 29 and 30 insert:

SUMMARY OF THE INVENTION

Page 5, between numbered lines 37 and 38 insert:

ILLUSTRATIVE EXAMPLES

In The Claims:

1. (Amended) A method for the production of an immunostimulant milk product, said method comprising carrying out bioconversion on a milk substrate with the aid of a *Bifidobacterium* culture by keeping said substrate in contact with said culture, under conditions which are unfavorable to fermentation by *Bifidobacterium*.

2. (Amended) The method as claimed in claim 1, wherein the milk substrate and the *Bifidobacterium* are brought into contact at the rate of 1×10^7 to 1×10^9 CFU per ml of milk substrate, and the final *Bifidobacterium* population at the end of the bioconversion reaction is 1×10^5 to 1×10^9 CFU per ml of product.

3. (Amended) The method as claimed in claim 1, wherein the pH of the milk substrate during the bringing into contact with the *Bifidobacterium* is 6.3 to 7 and the pH of the product at the end of the bioconversion reaction is 6 to 7.

4. (Amended) The method as claimed in claim 1, wherein the duration of contact between the milk substrate and the bacteria is 6 to 24 hours.

5. (Amended) The method as claimed in claim 1, wherein a *Bifidobacterium* culture comprising the *Bifidobacterium breve* strain deposited on May 31, 1999, under the number I-2219 at the CNCM, is used.

6. (Amended) A milk product obtained by the method as claimed in claim 1.

7. (Amended) The milk product as claimed in claim 6, wherein its pH is 6 to 7.

9. (Amended) The milk food as claimed in claim 8, wherein its pH is 6 to 7.5.

Please add new claim 10 as follows:

10. (New) The milk food as claimed in claim 9, wherein its pH is 6.5 to 6.9.

REMARKS

The above amendments are made to place this application into proper form for examination according to Unites States formalities requirements.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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"Express Mail" Mailing Label Number EL 822757828 US Date of Deposit: December 26, 2001 I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box Patent Application, Commissioner for Patents, Washington, DC 20231. Lorna Morehead	**CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on December 26, 2001. _____

Version With Markings to Show Changes Made:

1. (Amended) A method for the production of an immunostimulant milk product, said method comprising carrying out bioconversion [characterized in that bioconversion is carried out] on a milk substrate with the aid of a *Bifidobacterium* culture by keeping said substrate in contact with said culture, under conditions which are unfavorable to fermentation by *Bifidobacterium*.

2. (Amended) The method as claimed in claim 1, wherein [characterized in that] the milk substrate and the *Bifidobacterium* are brought into contact at the rate of 1×10^7 to 1×10^9 CFU per ml of milk substrate, and the final *Bifidobacterium* population at the end of the bioconversion reaction is 1×10^5 to 1×10^9 CFU per ml of product.

3. (Amended) The method as claimed in claim 1, wherein [either of claims 1 and 2, characterized in that] the pH of the milk substrate during the bringing into contact with the *Bifidobacterium* is [preferably] 6.3 to 7 and the pH of the product at the end of the bioconversion reaction is [preferably] 6 to 7.

4. (Amended) The method as claimed in claim 1, wherein [any one of claims 1 to 3, characterized in that] the duration of contact between the milk substrate and the bacteria is 6 to 24 hours.

5. (Amended) The method as claimed in claim 1, wherein [any one of claims 1 to 3, characterized in that] a *Bifidobacterium* culture comprising the *Bifidobacterium breve* strain deposited on May 31, 1999, under the number I-2219 at the CNCM, is used.

6. (Amended) A milk product[, characterized in that it is capable of being] obtained by the method as claimed in claim 1 [any one of claims 1 to 5].

7. (Amended) The milk product as claimed in claim 6, wherein [characterized in that] its pH is 6 to 7.

9. (Amended) The milk food as claimed in claim 8, wherein [characterized in that] its pH is 6 to 7.5[, preferably 6.5 to 6.9].

METHOD FOR THE PRODUCTION OF AN IMMUNOSTIMULANT MILK PRODUCT AND USES THEREOF

The present invention relates to the use of bifidobacteria for the preparation of immunostimulant milk foods which are suitable in particular for use as infant food: foods which may be in liquid or powdered form.

The genus *Bifidobacterium* belongs to the *Actinomycetaceae* family; it groups together Gram-positive bacilli which are strict anaerobes fermenting glucose by the fructose-6-phosphate phosphoketolase pathway. Their optimum pH for growth is between 6 and 7, and their optimum temperature for growth is between 37 and 40°C.

Bifidobacteria are part of the normal human intestinal flora and they are recognized to have numerous beneficial effects on health. It is in particular known that breast-fed unweaned babies who possess an intestinal flora in which bifidobacteria are predominant are more resistant to infections and have in particular a lower risk of diarrhea than unweaned babies fed with industrial milk preparations.

The role of bifidobacteria in this increased resistance to infections has not been completely elucidated. Various studies indicate that they possess an immunostimulant power which would involve polysaccharide substances associated with the bacterial wall, or secreted by the bacteria during anaerobic fermentation. GOMEZ et al., [FEMS Microbiol. Lett., 56, 47-52, (1988)] describe the immunomodulatory effect of exocellular fractions rich in polysaccharides produced by *Bifidobacterium adolescentis*; FR Application published under the number 2652590, in the name of Laboratoires OM, describes an immunopotentiative exopolymer of a polysaccharide nature produced by *Bifidobacterium infantis longum*; HOSONO et al., [Biosci. Biotech. Biochem., 61, 312-316 (1997) and Bioscience Microflora, 17, 97-104, (1998)] describe

immunopotentiator polysaccharides produced by various *Bifidobacterium* species. The immunomodulatory action of bifidobacteria also manifests itself by the regulation of the intestinal microflora, in particular at the expense of the development of pathogenic bacterial species. ROMOND et al., [Anaerobe, 3, 137-143, (1997), and J. Dairy Sci., 81, 1229-1235, (1998)] thus describe glycoprotein-rich fractions produced by *Bifidobacterium breve* under anaerobic fermentation conditions, and inducing *in vivo* a regulatory effect on the intestinal microflora.

Numerous products fermented by bifidobacteria, optionally combined with other lactic acid bacteria, exist on the market whose ingestion make it possible to benefit from the immunostimulant effects of bifidobacteria and of their fermentation products.

In the case of infant nutrition, however, they have the disadvantage of being too acidic and of having, in particular in the case of powdered products, a nonhomogeneous appearance after reconstitution, because of the coagulation of the milk proteins by the acidity generated during fermentation. They are therefore sometimes poorly accepted by the child or by the mother.

However, the inventors have now discovered that the production, by bifidobacteria, of substances having immunostimulant properties could be carried out without fermentation, and therefore without acidification of the final product.

The subject of the present invention is a method for the production of an immunostimulant milk product, characterized in that bioconversion is carried out on a milk substrate with the aid of a *Bifidobacterium* culture by keeping said substrate in contact with said culture, under conditions which are unfavorable to fermentation by *Bifidobacterium*.

The expression "conditions which are unfavorable to fermentation by *Bifidobacterium*" defines conditions under which the acidification of the medium

by *Bifidobacterium* does not exceed 0.5 pH units during 8 hours of incubation for an initial inoculation of 1 to 5 × 10^7 CFU per ml. They can be easily determined by persons skilled in the art with the aid of simple trials, by varying in particular the aeration of the culture medium, its osmotic pressure and/or the culture temperature, and by measuring the pH at the beginning and at the end of the culture.

For a large number of *Bifidobacterium* strains, such conditions may be obtained in particular by:

- keeping under aerobic conditions, for example with stirring;
- keeping the medium at an osmotic pressure corresponding to a water activity (AW) of 0.93 to 0.97;
- keeping at a temperature of 40 to 48°C;

as well as combinations of these various conditions.

The milk substrate and the *Bifidobacterium* may be brought into contact at the rate of 1 × 10^7 to 1 × 10^9 CFU per ml of milk substrate, and the final *Bifidobacterium* population at the end of the bioconversion reaction is 1 × 10^5 to 1 × 10^9 CFU per ml of product.

The pH of the milk substrate during the bringing into contact with the bacteria is preferably 6.3 to 7 and the pH of the product at the end of the bioconversion reaction is preferably 6 to 7.

Depending on the conditions used, the duration of contact between the milk substrate and the bacteria will be 6 to 24 hours.

The milk substrate may be milk, or any milk-based medium; this may be for example a milk concentrate, a base for infant milk food, a base for yogurt, and the like.

It is possible to add to the milk-based medium the ingredients necessary for producing the product ready for consumption which it is desired to obtain. If, for example, it is desired to obtain a milk food

for unweaned babies, lactose, maltodextrins, minerals, vitamins, fat, and ingredients which make it possible to reconstitute the composition of breast milk, will be added. If desired, the fat is incorporated and then homogenized with the solution so as to obtain a stable emulsion.

The *Bifidobacterium breve* strain which is particularly suitable for carrying out the invention, was deposited in accordance with the Budapest Treaty, on May 31, 1999, under the number I-2219 at the CNCM (Collection Nationale de Cultures de Microorganismes) held by Institut Pasteur, 25 rue du Docteur Roux, in Paris.

This strain has the following characteristics:

morphology: short bacilli with rare Y and V shapes

metabolism: anaerobiosis; production of L-(+)-lactic and acetic acids.

fermentation of sugars: glucose, galactose, fructose, maltose, sucrose, lactose, esculin, ribose, mannitol, sorbitol, D-raffinose, melibiose.

The subject of the present invention is also a liquid milk product characterized in that it may be obtained using a method in accordance with the invention.

This product preferably has, at the end of the bioconversion reaction, a pH of 6 to 7.

By way of comparison, the prior art products obtained by fermentation by *Bifidobacterium* have, at the end of fermentation, a pH of 4 to 4.6.

This product may be consumed as it is, or may be subjected to various treatments whose nature varies according to the ready-to-eat product which it is desired to obtain. It may, for example, be supplemented with texturing agents, flavoring agents, vitamin or mineral supplements, fat and the like, if these were not added to the original medium. It may also be concentrated or diluted.

A milk product in accordance with the invention can serve as base for the preparation of fresh milk foods.

Advantageously, it may also be used for the preparation, by sterilization and/or dehydration, of foods with long shelf life. Indeed, it preserves its immunostimulant properties even in the absence of live bacteria, for example after desiccation and UHT sterilization.

The present invention also covers the fresh, sterilized or dehydrated milk foods obtained from a milk product in accordance with the invention.

It also covers the reconstituted milk foods obtained by adding water to the dehydrated milk foods in accordance with the invention.

The (fresh, sterilized or reconstituted) milk foods in accordance with the invention generally have a pH of 6 to 7.5, preferably of 6.5 to 6.9.

Unlike the foods resulting from fermentation by *Bifidobacterium* which are known in the prior art, the milk foods in accordance with the invention are not acidic, and contain the milk proteins in soluble, noncoagulated form. Upon addition of water to the dehydrated milk foods in accordance with the invention, it is thus possible to obtain a homogeneous product, without precipitation or phase separation.

The milk foods in accordance with the invention, by virtue of their immunostimulant effect, confer protection against microbial and viral infections comparable to that of the foods resulting from fermentation by *Bifidobacterium* which are known in the prior art, without having the disadvantages thereof in terms of modification of the taste and of the appearance of the product. They are particularly suitable for use as infant food, and in particular as food for unweaned babies, but they may also be used as food for subjects of all ages.

The present invention will be understood more clearly with the aid of the additional description

which follows, which refers to examples of preparation of milk products in accordance with the invention.

EXAMPLE 1: MANUFACTURE OF A POWDERED DIETETIC MILK PREPARATION FOR UNWEANED BABIES, WITH IMMUNOSTIMULANT ACTIVITY

A milk concentrate is prepared whose composition, expressed in g per 100 g of dry matter, is the following:

Milk proteins (80% casein and 20% serum proteins)	13
Vegetable fat	25.5
Lactose	42.25
Maltodextrins	16
Minerals	3
Vitamins	0.25

The vegetable fat is added to a skimmed cow's milk, heated to 75°C. Homogenization is performed at the same temperature in 2 stages, the first under 200 kgs/cm^2, the second under 50 kgs/cm^2. The lactose and the maltodextrins, previously dissolved in water, are then added, followed by the solutions of vitamins and minerals.

The final mixture is pasteurized at 115°C and then concentrated by evaporation to 48% dry matter.

The concentrate, cooled to 37°C, is then inoculated at the rate of 5% with a *B. Breve* I-2219 culture containing 10^9 bacteria/ml. The initial pH is 6.15 and the osmotic pressure is 0.96.

After incubating for 8 h at 37°C, in a tank under air with periodic stirring for 10 minutes every 2 hours, the pH is 6.1 and the *B. Breve* population is 10^6 bacteria/ml. The Dornic acidity is 48°D.

The concentrate is spray-dried. The powder obtained, when added to water in an amount of 140 g per liter of water, makes it possible to obtain a reconstituted milk which possesses the following characteristics: pH 6.6, Dornic acidity 12°D; liquid milk appearance with no curd grains.

EXAMPLE 2: MANUFACTURE OF A READY-TO-USE, UHT-STERILIZED AND ASEPTICALLY PACKAGED DIETETIC MILK PREPARATION FOR UNWEANED BABIES, WITH IMMUNOSTIMULANT ACTIVITY

A mixture is prepared whose composition (in g/liter) is the following:

Proteins	21
Fat	24
Carbohydrates	83
Minerals	5
Vitamins	0.45

This mixture is prepared from the following ingredients (per 100 liters of finished product):

- 58 liters of skimmed milk,
- 2.4 kgs of fat,
- 4.7 kgs of lactose,
- 0.7 kgs of maltodextrins,
- 0.3 kg of vitamins,
- 0.05 kg of mineral complex.

The milk is heat-treated beforehand using the UHT system to a temperature of 115 to 120°C.

The fat is incorporated into the milk which has been cooled to 70°C and homogenization is performed in 2 stages, 200 kgs during the 1st stage, 50 kgs in the 2nd stage.

The mixture is cooled to 37-38°C, and then inoculated at 1.5% with a CNCM I-2219 culture containing 1 to 5 $\times$ 10^9 bacteria/ml.

Incubation is carried out at 37°C for 8 hours under conditions identical to example 1 above, followed by cooling to 5°C.

The pH of the product is 6.3 and the *B. Breve* population is 3 $\times$ 10^7 bacteria/ml. The Dornic acidity is 23°D.

The remainder of the ingredients is dissolved in 50 liters of water approximately and then added to the product obtained at the end of the incubation.

The mixture thus produced is subjected to a UHT treatment at 140°C for 6 to 7 seconds before being aseptically packaged.

EXAMPLE 3: IMMUNOSTIMULANT EFFECT OF MILK PRODUCTS IN ACCORDANCE WITH THE INVENTION

The immunostimulant effect of the milk preparations in accordance with the invention was studied as follows:

- by the development of fecal flora on mice with human flora;
- by the regulation of the phenomenon of translocation on monoxenic mice with *Clostridium perfringens*.

Studies of the development of the fecal flora in mice with human flora:

The mice are of the adult C3H line with human flora.

This is the G1 generation, the G0 generation being axenic mice associated in adulthood with the human flora.

- Number of mice per group: 6
- Number of trials: 2 per product.

The mice are kept for 1 week in the same cage and then divided 6 to a cage.

The age of the mice at the beginning of the trials is 8 weeks minimum and 11 weeks maximum.

The following will be monitored in the fecal flora:

- bifidobacteria
- *Bacteroides fragilis*
- spores of *Clostridia*
- optionally spores of *Cl. perfringens*

Microbiological techniques

The fecal sample is collected immediately before use, aseptically weighed and diluted in pre-reduced Ringer's solution (diluted one quarter and supplemented with cysteine hydrochloride at 0.3 g/l).

Enumeration of bifidobacteria and *Bacteroides fragilis* on pre-reduced BEERENS and BBE media

inoculated directly and incubated under anaerobic conditions.

For the test for *Clostridium* spores:

- the suspensions are heated for 10 minutes at 75°C and inoculated on Columbia agar supplemented with glucose (5 g/l) and cysteine hydrochloride (0.3 g/l) and incubated for 5 days,

- the *Clostridium* colonies are identified by their morphology and a negative catalase reaction. The cell morphology is determined after Gram staining 3.

The results obtained with a control milk preparation which had been inoculated with the ferment CNCM I-2219 and administered immediately are illustrated by table I below (contact time = 0)

Table I

	T 0	T 7 days	T 15 days
Bifidobacteria	8.2 ± 0.3	9.3 ± 0.1	8.6 ± 0.1
Bacteroides fragilis	7.2 ± 0.5	9.3 ± 0.1	9.2 ± 0.1
Clostridium	4.3 ± 0.1	5.1 ± 0.5	6.7 ± 0.3

The results are expressed in log and the figures represent the mean of the results for the 6 mice; a significant increase in *Bacteroides fragilis* and *Clostridia* is observed, hence an infectious risk.

The results obtained with a milk preparation in accordance with the invention, inoculated and having been subjected to an 8-hour contact at 37°C with CNCM I-2219, are illustrated by table II below.

Table II

	T 0	T 7 days	T 15 days
Bifidobacteria	7.1 ± 0.1	11 ± 0.5	10.3 ± 0.8
Bacteroides fragilis	8 ± 0.2	7.9 ± 0.3	nd < 4.7 log
Clostridium	3.9 ± 0.3	4.4 ± 0.2	4 (1 mouse) 5 others: absence
C. perfringens	3.7 ± 0.9	nd	nd

nd: not determined

A 2.5 log increase in bifidobacteria and a very large reduction in *Bacteroides* and *Clostridia* is

observed, relative to the control, in particular after administering for 15 days.

Studies on monoxenic mice with *Clostridium perfringens*:

Objective: to verify the influence of the products in accordance with the invention on the dissemination of intestinal bacteria in various organs.

Experimental condition: axenic mice (age = 8 weeks) kept in a sterilized isolating unit, fed on RO3 base sterilized by irradiation.

Products tested:

- ultrapure water sterilized by autoclaving
- ultrapure water sterilized by autoclaving supplemented with a preparation in accordance with the invention (PII) at the rate of 14 g (weight of powder) per 100 ml of water.

These solutions are prepared in a sterile manner daily and given *ad libitum* to the mice for 6 days. At the end of this period, *C. perfringens* strain LAB (human intestinal origin) is inoculated at the rate of 3.5 to 4.5 log CFU per mouse. The implantation and dissemination of *Clostridium perfringens* in the lymphoid organs are measured by sacrificing two mice per group 24, 48 hours, 4 days and 7 days after inoculation. The counts are performed by the most probable number method with three tubes in LS medium (incubation 46°C 24-48 hours).

The results are illustrated by table III below:

Table III

	D1		D2		D4		D7	
	PII	Water	PII	water	PII	water	PII	water
Ileum proximal	2	2	0	1	0	2	2	2
Median	2	0	0	1	0	2	2	2
Distal	2	0	0	1	0	2	2	2
Cecum	2	0	0	2	1	2	2	2
Colon	2	2	0	2	2	2	2	2
Peyer's patches	1	1	0	0	1	2	2	2
Mesenteric ganglia	0	2	0	0	1	2	1	2
Bacteriemia	0	0	0	0	0	0	0	0
Spleen	0	1	0	2	0	2	1	2
Liver	0	0	0	0	1	2	2	2
Kidney	0	1	0	2	1	2	1	2
Lung	0	0	0	0	0	2	0	0

Legend to table III:

0 = low implantation/dissemination

1 = average implantation/dissemination

2 = high implantation/dissemination

The following are observed:

- a 24-hour delay in implantation of *C. Perfringens* after administration of the product in accordance with the invention;

- low dissemination in the lymphoid organs in mice which consumed the product in accordance with the invention (PII).

These results show that the preparations in accordance with the invention regulate the dissemination of *Clostridium perfringens* in the lymphoid organs.

CLAIMS

1. A method for the production of an immunostimulant milk product, characterized in that bioconversion is carried out on a milk substrate with the aid of a *Bifidobacterium* culture by keeping said substrate in contact with said culture, under conditions which are unfavorable to fermentation by *Bifidobacterium*.

2. The method as claimed in claim 1, characterized in that the milk substrate and the *Bifidobacterium* are brought into contact at the rate of 1×10^7 to 1×10^9 CFU per ml of milk substrate, and the final *Bifidobacterium* population at the end of the bioconversion reaction is 1×10^5 to 1×10^9 CFU per ml of product.

3. The method as claimed in either of claims 1 and 2, characterized in that the pH of the milk substrate during the bringing into contact with the *Bifidobacterium* is preferably 6.3 to 7 and the pH of the product at the end of the bioconversion reaction is preferably 6 to 7.

4. The method as claimed in any one of claims 1 to 3, characterized in that the duration of contact between the milk substrate and the bacteria is 6 to 24 hours.

5. The method as claimed in any one of claims 1 to 3, characterized in that a *Bifidobacterium* culture comprising the *Bifidobacterium breve* strain deposited on May 31, 1999, under the number I-2219 at the CNCM, is used.

6. A milk product, characterized in that it is capable of being obtained by the method as claimed in any one of claims 1 to 5.

7. The milk product as claimed in claim 6, characterized in that its pH is 6 to 7.

8. A milk food obtained from a product as claimed in claim 7.

9. The milk food as claimed in claim 8, characterized in that its pH is 6 to 7.5, preferably 6.5 to 6.9.

French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119(a)-(d) ou § 365(b) du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur ou, en vertu du Titre 35, § 365(a) du même Code, sur toute demande internationale PCT désignant au moins un pays autre que les Etats-Unis et figurant ci-dessous et, en cochant la case, j'ai aussi indiqué ci-dessous toute demande étrangère de brevet, tout certificat d'inventeur ou toute demande internationale PCT ayant date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

I hereby claim foreign priority under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below, and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign application(s)
Demande(s) de brevet antérieure(s) dans un autre pays.

(Number) (Numéro)	(Country) (Pays)	(Day/Month/Year Filed) (Jour/Mois/Anné de dépôt)	Priority claimed Droit de priorité revendiqué Yes Oui	No Non
99/08691	**France**	**July 6, 1999**	☒	☐
			☐	☐

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 119(e) du Code des Etats-Unis, de toute demande de brevet provisoire effectuée aux Etats-Unis et figurant ci-dessous.

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis, ou en vertu du Titre 35, § 365© du même Code, de toute demande internationale PCT désignant les Etats-Unis et figurant ci-dessous et, dans la mesure où l'objet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande antérieure américaine ou internationale PCT, en vertu des dispositions du premier paragraphe du Titre 35, § 112 du code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la brevetabilité, comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la demande antérieure et la date de dépôt de la demande nationale ou internationale PCT de la présente demande :

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)	(Status) (patented, pending,abandoned) (Statut) (breveté, en cours d'examen, abandonné)

Je déclare que par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique ;et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la section 1001 du Titre 18 du Code de Etats-Unis, et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hebery declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declaration and Power of Attorney for Patent Application
Déclaration et Pouvoirs pour Demande de Brevet
French Language Declaration

En tant l'inventeur nommé ci-après, je déclare par le présent acte que :

Mon domicile, mon adresse postale et ma nationalité sont ceux figurant ci-dessous à côté de mon nom.

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) de l'objet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée

et dont la description est fournie ci-joint à moins

☐ ci-joint

☐ a été déposée le

sous le numéro de demande des Etats-Unis ou le numéro de demande international PCT

et modifiée le

(le cas échéant).

Je déclare par le présent acte avoir passé en revue et compris le contenu de la description ci-dessus, revendications comprises, telles que modifiées par toute modification dont il aura été fait références ci-dessus.

Je reconnais devoir divulguer toute information pertinente à la brevetabilité, comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations.

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed an for which a patent is sought on the invention entitled

METHOD FOR THE PRODUCTION OF AN IMMUNOSTIMULANT MILK PRODUCT AND USES THEREOF

the specification of which :

☐ is attached hereto.

☒ was filed on **December 26, 2001**

as United States Application Number or PCT International Application Number **10/019,872**

and was amended on

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations,§ 1.56.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocats(s) et/ou agent(s) suivant(s) pour qu'ils poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire s'y rapportant avec l'Office des brevets et des marquees : (mentionner le nom et le numéro d'enregistrement).

POWER OF ATTORNEY : As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to persecute this application and transact all bussiness in the Patent and Tradémark Office connected therewith : (list name and registration number)

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(Supply similar information and signature for third and subsequent joint inventors.)

French Language Declaration

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Signature de l'inventeur Date	Sixth inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse Postale	Post Office Address

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

Supply similar information and signature for third and subsequent joint inventors.)